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TITLE : BLOOD CELL INCREASING AGENT

ABSTRACT : PURPOSE: To obtain a blood cell increasing agent useful as a therapeutic agent for dysfunction of hematopoiesis due to suppression of myeloma function by including cathepsin L having action capable of strongly promoting the proliferation of megakaryocyte and derived from a human fibroblast cell as an effective component.

CONSTITUTION: This blood cell increasing agent contains cathepsin L as an effective component. The agent can especially be utilized in treatment of diseases accompanied by decrease of a megakaryocytic cell and treatment for thrombocytopenia. As the cathepsin L, especially, a cathepsin L derived from a human fibroblast cell is preferably used. For example, cathepsin L is obtained by separation and purification of a supernatant of cultured human fibroblast cells or separation and purification of a cell extract or the supernatant of the cultured cells which are prepared by a gene recombinant technique using a DNA corresponding to the hematopoietic factor, or by purification and separation from body fluid component of milk of a transgenic animal obtained by injecting the cDNA corresponding to the hematopoietic factor into a fetal embryo through a proper vector system.

* NOTICES *

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2. **** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] this invention relates to the increase agent in a blood cell which contains the new hematogenous factor useful for medical treatment and these hematogenous factors of a disease, such as platelet ******, as an active principle.
[0002]
[Description of the Prior Art] The platelet has the important role committed in promotion in the process of the thrombus formation for stopping bleeding at the time of bleeding, or blood coagulation. Although a platelet is produced from a bone marrow megakaryocyte, the humoral factor which acts on the process is divided into the megakaryocyte colony stimulating factor (Megakaryocyte-colony stimulating factor:Meg-CSF) which acts in early stages of specialization, and the thrombopoietin (Thrombopoietin:TPO) which acts on the **** precursor cell which matured to some extent or a megakaryocyte amplifier (Megakaryocyte-potentiator:POT). In spite of the research for the past 20 years or more, the factor specifically concerned with megakaryocyte platelet system hemopoiesis is not yet identified, but it waits eagerly for the discovery and application for a long time.
[0003] In recent years, it is shown clearly that it participates in a platelet hematogenous system, and some of separated cytokines attract attention.
[0004] Meg-CSF activity is detectable by a stimulus of the cell division of a megakaryocyte precursor cell (megakaryocyte colony-forming unit:CFU-Meg), or the increase in colony size, and by in vitro, if a man or the bone marrow cell of a mouse is cultivated in soft agar or a methyl cellulose, it will be measured as activity in which a megakaryocyte colony is made to form. As Meg-CSF now reported, it is interleukin 3 (IL-3) and a granulocyte-macrophage colony stimulating factor (GM-CSF), and considers as the factor which Stem cell factor (SCF=c-kit ligand) also commits to a megakaryocyte precursor cell.
[0005] On the other hand, the size and the amount of DNA of a cell in a megakaryocyte colony are measured, and Meg-POT activity is detected by making the increase in the size of a megakaryocyte, the increase in the amount of DNA of a megakaryocyte, or the increase in the amount of megakaryocyte enzymes (acetylcholineaterase) into an index, cultivating a man or the bone marrow cell of a mouse in soft agar or a methyl cellulose in in vitro, and carrying out the fixed quantity of the acetylcholineaterase under liquid culture. As a cytokine with Meg-POT activity, IL-6, IL-7, IL-11, erithropoietin (EPO), a macrophage colony stimulating factor (M-CSF), Leukemia inhibitory factor (LIF), etc. are reported.
[0006] Specifically, Meg-CSF and a Meg-POT activity-measurement method are Tanaka et al. It is detailed to a report (Blood, 80, 1743-1749, 1992), and the grade of an operation of various cytokines is stated to the total theory (experiment medicine, 10, 365-376, and 1992) of Kahoku.
[0007] As the factor which works to the megakaryocyte platelet system hemopoiesis other than the known cytokines which have Meg-CSF or Meg-POT activity described above The molecular weight 15000 of the man embryo nephrocyte origin [measurement by the polyacrylamide gel electrophoresis (SDS-PAGE) containing a sodium dodecyl sulfate], The megakaryocyte promoter (JP,63-239298,A) of pI5.1, the molecular weight 24000 of the man lung cancer cell origin, the megakaryocyte system colony stimulating factor of pI 4.5-5.5 (WO 90/03397), The molecular weight 25000**8000

(measurement by gel filtration) of the man fiber blast cell origin, the megakaryocyte amplifier (JP,4-29550,A) of pI>9 -- the platelet growth factor (Nikkei BAIOTEKU, the June 8, 1992 issue) of the molecular weight 50000 (homodimer of a monomer 25000) of the man urine origin etc. is reported further

[0008] However, it is not proved whether it is still unknown whether it is Meg-CSF or Meg-POT on which these factors are originally functioning physiologically, and it may become useful medicine or a useful diagnostic drug on industry and medical treatment.

[0009]

[Problem(s) to be Solved by the Invention] As mentioned above, some factors which have Meg-CSF or Meg-POT activity conventionally have been found out as a hematogenous factor which is made to proliferation-promote or amplification promote a megakaryocyte, and makes a thrombocytosis principal action. However, the present condition is having not made clear the superiority or inferiority on the usefulness on the industry of these factors, and medicine, and use between each factor, either. Therefore, search of the matter which is essentially working as Meg-CSF or a Meg-POT active factor in original the living body is continued, and discovery and its use of such an essential factor are left behind as an important technical problem which should be solved on industry and medicine.

[0010] It is in offering the increase agent in a blood cell which contains the hematogenous factor which has useful megakaryoblast amplification activity on industry and medicine, and this hematogenous factor as an active principle that this invention should solve this technical problem.

[0011]

[Means for Solving the Problem] This invention persons completed this invention, as a result of repeating research wholeheartedly to find out the new megakaryoblast amplifier which has the operation which promotes amplification of a megakaryocyte powerfully. That is, according to this invention, it is the increase agent in a blood cell which makes an active principle the cathepsin L which has megakaryoblast amplification activity.

[0012] the Homo sapiens cathepsin L is already known as a kind of a thiol protease -- having -- **** (Mason et al., Biochemical Journal, 240, 373-377, and 1986) -- an elastin -- the operation (Johnson et al., Journal of BiologicalChemistry, 261, 14748-14751, and 1986) which inactivates resolution (Mason et al., Biochemical Journal, 233, 925-927, and 1986) and an alpha1-protease inhibitor is reported

[0013] The pre pro object of the Homo sapiens cathepsin L consists of 333 amino acid residues (molecular weight 38000), and, as for a precursor (pro object), one to amino terminus 17 residue is removed. That is, an N-terminal-amino-acid array is as the array number 1 of an array table including 18 -333 residue (it is molecular weight 36000 at 316 amino acid residues) (Joseph et al., J.Clinic.Investigation, 81, 1621-1629, and 1988). Among these, 18 -113 residue is called acouchi BESHON peptide, and a cathepsin H heavy chain and 292 to 333 residue of 114 -288 residue are cathepsin L chains.

[0014] However, the physiological activity as a hematogenous factor of these polypeptides component was not known at all conventionally, but the megakaryoblast amplification activity was clarified for the first time by this invention. That is, this invention relates to the increase agent in a blood cell which contains the cathepsin L which has megakaryoblast amplification activity as an active principle.

[0015] the cathepsin L of this invention -- a precursor (18 -333 residue), a mature object (114 -333 residue), an H chain (114 -288 residue), and an L chain (292 -333 residue) -- although all are contained, a precursor is used preferably

[0016] Since it can obtain by the cell culture, and also cloning of the cDNA is already carried out and all base sequences are determined (Joseph et al., Journal of Clinical Investigation, 81, 1621-1629, 1988), the cathepsin L used in this invention can apply the so-called gene modification technology, and can also acquire it as recombinant protein.

[0017] That is, this hematogenous factor is obtained using cDNA corresponding to the refining separation from a Homo sapiens cultured cell culture supernatant, or this hematogenous factor by [of body fluid components, such as milk of the so-called transgenic animal which poured in the refining separation from the extract or cell culture supernatant liquid of the cell produced by the so-called

gene modification technology, and cDNA corresponding to this hematogenous factor by the suitable vector system, and was further obtained by the embryo germ,] carrying out refining separation.
[0018] Homo sapiens cultured cells are a corpuscle system cell, the fibroblast, a bone marrow stromata cell, and fibroblast preferably, although either of the various kinds of normal-tissue origin cells or established cell line which has the capacity which produces this hematogenous factor is applicable.
[0019] When preparing this hematogenous factor using gene modification technology, microorganisms, such as insect cells, such as mammalian cells, such as a CHO (Chinese hamster ovary cell) cell and mouse C127 cell, a silkworm, and a cutworm, Escherichia coli, a Bacillus subtilis, and yeast, etc. can be used as a host cell. Furthermore, when making a transgenic animal into a host, a mouse, a rat, a hamster, a rabbit, a goat, a sheep, a swine, a cow, etc. can be used.
[0020] Thus, refining separation of this hematogenous factor can be carried out with various chromatographies by using the cell culture supernatant liquid containing this prepared hematogenous factor, a polypide extract, a biomass extract, living body body fluid, etc. as a raw material. Although any are sufficient as the chromatography to be used as long as it has compatibility in this hematogenous factor, they are the column which uses a silicon dioxide (silica) and calcium phosphate as adsorption material, the column which makes a ligand a heparin, coloring matter, and the amount of canals, a metal chelate column, an ion exchange column, a gel filtration column, etc., for example.
[0021] This refined hematogenous factor can be preferably used as a therapeutic drug of a thrombocytosis use, although it can use as a treatment agent of the hematogenous-functions incompetence by bone marrow functional suppression. For example, it can use for the treatment and prevention of the thrombocytopenia after anticancer agent medication, the thrombocytopenia after radiation therapy, the thrombocytopenia by the megakaryocyte amplifier deficit, the thrombocytopenia of hypoplastic anemia, the thrombocytopenia after a bone marrow transplantation, and the thrombocytopenia of an autoimmune disease. Moreover, it can use as the treatment of a part of leukemia which is accompanied by reduction of a megakaryocyte system cell, the treatment of an osteomyelodysplasia syndrome, the proliferation agent at the time of in vitro prior cultivation of the marrow cell for transfusion and also the alternative agent of platelet transfusion, or an adjuvant.
[0022] that the hematogenous factor of this invention remains as it is, or the very thing -- as the physic constituent mixed with the well-known support permitted in pharmacology, the excipient, etc. -- taking orally -- or a medicine can be parenterally prescribed for the patient
[0023] As dosage forms for internal use, a tablet, the pilule, a capsule, a granule, the syrup, an emulsion, the suspension, etc. are specifically mentioned. these dosage forms -- the very thing -- it is manufactured by the well-known method and the support or the excipient usually used in a tablet field is contained For example, as the support for tablets, and an excipient, a lactose, a maltose, a saccharose, starch, a magnesium stearate, etc. are mentioned.
[0024] As dosage forms for parenteral administration, an ointment, the injection, a poultice, the paint, a suppository, a pernasal absorbent, a transpulmonary absorbent, a percutaneous absorption agent, etc. are mentioned, for example. a solution tablet -- the very thing -- the sterile solution usually used for the injection prepares a well-known method, for example, the hematogenous factor of this invention, by the dissolution, and it may be prepared by the extract, where it emulsified further and embedding is carried out to a liposome, suspension and as a solid-state tablet -- the very thing -- a mannitol, a trehalose, a sorbitol, a lactose, a glucose, etc. are added to a well-known method, for example, the hematogenous factor of this invention, as an excipient, and it may be prepared as a freeze-drying object as a gelling agent -- the very thing -- it may be prepared where a well-known method, for example, the hematogenous factor of this invention, is dissolved in thickeners, such as a glycerol, a polyethylene glycol, a methyl cellulose, and a carboxyl methyl cellulose
[0025] Also in which tablet, alcohol, sugar-alcohol, an ionicity interface slippage agent, a nonionic surfactant, etc. can be added in the range which can add a human serum albumin, a human immunoglobulin, the alpha2 macroscopic Globulin, amino acid, etc. as a stabilizing agent, and does not spoil the physiological activity of the hematogenous factor of this invention as a dispersant or an absorption accelerator.

[0026] Although the effective dose and the number of times of medication of a hematogenous factor of this invention change also with the properties or the degrees of critical of medication dosage forms, a medication rate, a patient's age, weight, and the symptom that should be treated, they can usually prescribe 0.1-10mg for the patient for 0.01-100mg per one adult in 1 time or several steps preferably.

[0027]

[Example] Although an example is hereafter shown in order to explain this invention to a detail more, this invention is not limited to these.

[0028] Seeding of the example 1 Homo-sapiens fibroblast was carried out to 1l. of MEM which contains a new calf blood serum 5% by 1x106 cell/ml, and it cultivated for 37 degrees C and five days, having made the micro carry ("Cytodex 1", Pharmacia Corp.) paste 0.3%, and agitating by the 2l. glass cultivation tub. Then, it exchanged for 1l. of non-blood serum MEM culture media, and Homo sapiens interferon beta was added by 100 international units / ml. 24 hours after, (Poly I):poly (C) was further added by ml in 10microg /, it replaced by the MEM culture medium containing a little methyl cellulose the 2 hours after, and cultivation was continued for six days after that. After the cultivation end, after making a microcarrier sediment, the supernatant liquid was moved to another container and it considered as the refining undiluted solution.

[0029] After pouring 5l. of refining undiluted solutions from which it filtered with the filter and the impurity was removed in the silica bead column (50ml, 5000nm pore, and Fuji DEBISON) and washing by 20mM phosphate buffer solution (PB) (pH 7)200ml, it was eluted with 20mM hydrochloric acid (pH 2). After adding the 0.3M phosphoric-acid hydrogen disodium solution, adjusting to pH 6.4 and carrying out centrifugal separation (3000rpm, 30 minutes) of the precipitate to 100ml of proteinic peak fractions, it dipped in the "Heparin-Cellulofine" column (5ml and Chisso Corp.).

[0030] 110ml of bypassing fractions was condensed to 15ml by "Centricut" (the cut-off molecular weight 10000 and Kurabo Industries, Ltd.). Gel filtration was performed for this concentration liquid in 3 steps in Superdex pg 200 column (2.6x60cm, Pharmacia Corp.). A developing solution is 0.3M. 20mM phosphate buffer solution (pH 7.4) containing NaCl was used. 90ml of activity fractions detected by the measuring method of the megakaryoblast amplification activity mentioned later was injected into C4 antiphase column (1x25cm and Vydac), and the Homo sapiens cathepsin L was eluted by the concentration gradient-elution method of the water/acetonitrile which contains a torr gifblaar poison (pH 2) 0.1%. It is Speed Vac in 2ml of activity fractions. Reduced pressure hardening by drying was carried out with the concentration machine, and it melted to distilled water of 100microl.

[0031] Next, it is Laemmli about this concentration activity fraction. According to the method (Nature, 227, 680-685, 1970), polyacrylamide gel electrophoresis (PAGE) which contains a sodium dodecyl sulfate (SDS) under nonreduction conditions was performed, and it refined further. after migration and SDS-PAGE gel -- 2mm width of face -- slicing -- per [the piece (1x2x4mm) of a slice] -- it was immersed by 0.5ml phosphate buffered saline (PBS, pH 7.4) 4 degrees C and overnight, and the protein in gel was eluted The megakaryoblast amplifier activity in the eluate of an activity fraction was 200 units / ml.

[0032] When again the fraction which has megakaryoblast amplifier activity and it carried out the argentation, the band single in the position of molecular weight 37000**3000 was detected. [under nonreduction conditions] When the amino acid sequence was analyzed for 5micro [of refining protein of this fraction] g by the protein sequencer (Applied Biosystams470 type), it checked that the amino acid sequence of ten amino terminuses was as the array number 1 of an array table, and was a Homo sapiens cathepsin L precursor.

[0033] Megakaryoblast amplifier activity was detected by the following methods. They are 1x104 cell / 0.5ml about the CMK cell (Sato et al., British Journal of Haematology, 72, 184-190, and 1989) which is a megakaryoblast system established cell line. Seeding was carried out to 24 well plastics plate by medium/well. Culture medium used RPMI1640 culture medium which contains a fetal calf serum (FCS) 5%. Subject sample 50microl was added to this, and it cultivated for 37 degrees C and five days. The number of cells and the cell diameter were measured with the cell-count machine (Coulter-counter ZM type) after cultivation, and the rate of an abundance ratio of the cell to which

the cell diameter amounted to 15 micrometers or more was computed. The rate of an abundance ratio of the subject group to the rate of an abundance ratio of an object group was computed as a megakaryocyte amplification activity ratio. The potency which increases 50% of activity ratios was made into one unit.

[0034]

[Effect of the Invention] Since the increase agent in a blood cell of this invention has megakaryoblast amplification activity, it can be used as a treatment agent of the hematogenous-functions incompetence by bone marrow functional suppression. It can use for the treatment of a disorder and the treatment of the thrombocytopenia accompanied by reduction of a megakaryocyte system cell preferably.

[0035]

[Layout Table]

array number: -- length [of one array]: -- ten topology: -- kind [of straight chain-like array]: -- protein fragmentation type: -- amino terminus fragmentation array Thr Leu Thr Phe Asp His Ser Leu Glu Ala1 5 10.

[Translation done.]